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Discrete regions of HIV-1 gp41 defined by syncytia-inhibiting affinity-purified human antibodies

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Objective: Fine mapping of HIV-1 gp41 fusion-critical sites.

Design and methods: Antibodies from human HIV-1-positive sera were affinity-purified on a panel of synthetic overlapping peptides spanning residues 526–682 of the extracellular portion of HIV-1 gp41. The syncytium-inhibiting capacity of the immunopurified antibodies and their differential reactivity on the synthetic peptides were tested.

Results: This approach enabled the identification of residues 583–591 (ARILAVERY), 595–599 (QQLLG), 603–609 (CSGKLIC) and 664–673 (ELLELDKWAS) as possibly involved in the fusion process. Reduction in the anti-ARILAVERY, anti-CSGKLIC and anti-ELLELDKWAS antibody titres and frequencies correlates with disease progression. Syncytia-inhibition capacity of sera did not correlate with the presence of high-titre antibodies reacting with any of the peptides tested, suggesting that most fusion-affecting antibodies are not directed towards gp41.

Conclusions: This strategy may be relevant for understanding the contribution of anti-gp41 antibodies in protecting against the pathogenic effects of the virus and in the design of an effective *env* vaccine.

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Keywords: HIV-1 gp41, epitope mapping, syncytia-inhibiting human serum antibodies, affinity-purification.

Introduction

The identification and characterization of epitopes involved in the essential functions of HIV-1 gp41 *env* protein that might play a role in the induction of protective immunity is fundamental for the development of an effective vaccine or therapeutic strategies.

Fine epitope mapping of gp41 transmembrane protein, using different experimental approaches, has hitherto enabled the identification of distinct functional sites, although conflicting results have been reported for some.

Neutralizable sites corresponding to residues 503–532 in the fusion domain [1,2] and residues 579–605 [3], 598–609 [4] and 647–671 [5] in the external domain have been identified; region 735–752 of the intracytoplasmic portion of gp41 has also been reported to induce *in vitro* neutralizing antibodies in rabbits [1,2,6]. An engineered poliovirus chimera expressing this epitope on its surface has been shown to elicit broadly reactive HIV-1-neutralizing antibodies in rabbits [7]. However, the definition of these sites as neutralizable is controversial, since antibodies to these regions devoid of neutralizing effects [3,8] and even with enhancing effects [9,10] have been reported. These discrepancies could depend on the different folding of the synthetic peptides used to cover these regions, as well as on the different assays used to assess the neutralizing activity of the antibodies. Moreover, the *in vivo* relevance of most of the reported results has yet to be clarified.

Epitope 598–609 (which contains two Cysteine residues critical for antigenic conformation, due to the creation of a cyclic structure via disulphide bond for-

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mation between residues 603 and 609) is immunodominant in HIV-seropositive individuals [4,11–13]. This and another immunodominant region between positions 644 and 663 have also been defined using human monoclonal antibodies [8,14]. The immunodominance of epitope 598–609 has been tentatively suggested to be the result of multiple priming of the host following repeated infections with viruses unrelated to HIV-1, but sharing similar epitopes [15].

Immunosuppressive sequences have also been identified in HIV-1 gp41. Residues 735–752 and 846–860 have been shown to inhibit the proliferative responses of T-cells and natural killer (NK) activity [16,17].

It is therefore of paramount importance to define the functional *in vivo* relevance of each region before considering it as a candidate component for a vaccine against HIV-1. Given the conserved nature of the gp41 protein [18–20], targeting antibodies to conserved neutralizable sites should provide effective protection against a broad spectrum of viral strains.

Most of the studies on anti-gp41-neutralizing antibodies have been performed in animal models. The aim of this study was to define HIV-1 gp41 discrete sites possibly involved in the fusion process identified by specific antibodies purified from the sera of infected individuals.

Materials and methods

Peptide synthesis

Peptides from the extracellular domain of gp41 were assembled using an automated Applied Biosystems 431A peptide synthesizer (ABI, Foster City, California, USA) following Merrifield's step-wise solid-phase procedure [21]. The carboxy-terminal amino acid was anchored onto 4-alkoxybenzyl alcohol resin using dicyclohexylcarbodiimide/4-dimethylaminopyridine [22]. The lateral protection groups used for trifunctional amino acids were the trifluoroacetic acid labile type, except for the S-acetamidomethyl derivative, which was used for Cysteine. All couplings were mediated by DCC/hydroxy- benzotriazole or by 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium exafluorophosphate [23]. When coupling incompletion was predictable (i.e., in long stretches of hydrophobic residues and in Arg residues), double coupling was performed. Cysteine, lysine and tyrosine were added at the amino terminus to enable conjugation to carrier protein and for iodination purposes. Cleavage of the assembled peptide from the solid support and removal of the side-chain-protecting groups was achieved by treatment with a mixture of water, phenol, thioanisole and ethandithiol in 85% trifluoroacetic acid for 90 min at room temperature. Crude peptide was precipitated and washed with tert-butylmethylether and directly purified to apparent homogeneity by low-pressure reverse-phase liquid chromatography (LPL on a semipreparative column filled with 15–20 µ silica (The Separation Group, Hesperia, Californ USA). Analytical high-pressure liquid chromatograp (HPLC) of the purified peptides was performed usi a TRI ROTAR-VI HPLC system (Jasco Internatior Company, Tokyo, Japan) in different eluting buff conditions. The purified peptides were further ch acterized for their correct amino-acid composition amino-acid analysis of the 6N HCl hydrolized a by circular dichroism spectroscopy. The purity of peptide preparations exceeded 95%. The numberi system used to designate the peptides is that of Wai Hobson *et al.* [24].

The coupling of peptides to solid support (CNE activated Sepharose 4B; Pharmacia LKB Biotechn ogy, Uppsala, Sweden) was performed followi the manufacturer's instructions. Peptide loading w 2–5 mg/ml gel.

Solid-phase anti-peptide ELISA

Microtitre plates were coated for 2 h with 100 ng pe tide in 50 mmol/l bicarbonate buffer (pH 9.5). Tl plates were saturated with 3% bovine serum alb min (BSA) in phosphate-buffered saline (PBS) for 2 at 37°C; serial dilutions of human sera or affinity-pu fied antibodies were incubated for 2 h at 37°C and, ter extensive washing, horseradish peroxidase-labell (HRP) rabbit anti-human immunoglobulin (Ig) w added for 1 h at 37°C. The enzymatic reaction w developed and read with a Titertek Multiscan (Flc Laboratories, Irvine, Scotland, UK).

Affinity purification of antibodies by Sepharose-linke peptides

Sera diluted in 2 ml PBS (250 µl) were incubat overnight at 4°C with 2–3 ml peptide-linked Sepharo under constant agitation. After extensive PBS was ing of the Sepharose columns, to eliminate the u bound fraction, bound antibodies were eluted in 10 r 0.2 mol/l glycine–HCl buffer (0.5 mol/l NaCl, pH 2.5 immediately buffered with 1 mol/l Tris, and then di lyzed against 1 : 10 diluted PBS and concentrated 1 times.

The IgG content of the eluted fractions after conce tration was evaluated as follows: the eluted fractio were assayed as competitors of the binding of HR labelled anti-human IgG antibodies (DAKO, Cope hagen, Denmark) to NH_4SO_4-precipitated normal h man serum IgG-coated microtitre plates. The conte of IgG was calculated on a calibration curve obtain using the same preparation of IgG used for the coatir (range, 1–3 µg/250 µl) as competitor.

Western blot analysis of purified antibodies

Immunoblotting was performed using standard met ods on commercial strips (Sorin Biomedica, Salu gia, Italy). Immunoblotted strips were incubated wi affinity-purified human antibodies (1 µg/ml) or dilute

sera (1 : 100), and then subjected to two further incubation steps with HRP rabbit anti-human Ig (DAKO) at 1:1000 (1 h) and Luminol (1 min), a diacylhydrazide, which develops a chemiluminiscent reaction upon oxidation (Amersham International, Amersham, England, UK). Autoradiography was performed according to standard methods.

Syncytia-inhibition assay

The fusion-inhibiting activity of sera and purified antibodies was evaluated as inhibition of syncytia formation between the lymphoblastoid cell lines 8E51 (LAV *pol*$^-$) and Molt-3. The assay [25] was performed as follows: 10^5 Molt-3 cells were incubated in V-shaped microtitre plates with 6×10^4 8E51 cells in 100 µl RPMI for 30 min at 37°C, in the presence of serial dilutions of human sera or affinity-purified antibodies. After incubation, the cells were sedimented by centrifugation and the pellets left for 4 h at 37°C, transferred to flat-bottomed plates and read under an inverted microscope with 40 × amplification. Only syncytia ≥10–100 cells were scored. Controls were performed with a pool of normal human sera. An anti-CD4 antibody (Becton Dickinson, Mountain View, California, USA) was used as the positive inhibition control. Two hundred large syncytia per well were counted in uninhibited controls.

Inhibition values at each dilution were obtained as follows: 100 − (number of syncytia in the presence of positive serum/number of syncytia in the presence of a pool of 10 normal human sera at the same dilution) × 100.

Results

Synthesis of peptides corresponding to residues 526–682 of HIV-1 gp41

Previous results from our laboratory [26] have shown that two recombinant fusion proteins, corresponding to residues 594–748 and 594–644 of HIV-1 gp41, are both fusion-inhibiting and induce fusion-inhibiting antibodies in mice and rabbits. To obtain a fine map of the fusion-relevant sites in this region, overlapping peptides from 20–30 residues covering the HIV-1 gp41 526–682 region [24] were synthesized (Fig. 1). The amino-terminal residues 526–594 and the carboxy-terminal residues 644–682 were included as reported to contain neutralizable sites [1–3,27] (V. Erfle, personal communication, 1993). The length of single peptides varied from 20 to 30 residues. Generally, long peptides are thought to assume spatial conformations closer to the folding of the corresponding epitope on native protein. Short polar hydrophilic amino tails, assumed not to interfere with the folding of single peptides, were added to increase solubility and to allow coupling to carrier proteins.

Recognition of gp41 526–682 region peptides by human seropositive sera

To define qualitative and quantitative differences in the spectrum of antibodies reactive to the 526–682 gp41 region and to analyse their neutralizing activity, sera from 48 patients in Centers for Disease Control (CDC) stages II (20 subjects), III (13 subjects) or IV (15 subjects) were tested using enzyme-linked immunosorbent assay (ELISA) on the panel of synthetic peptides listed in Fig. 1 and in the syncytia-formation-inhibition assay (Table 1).

Peptide ELISA screening of sera from the three groups of patients (a group of 10 normal subjects acted as controls) (Figs 2a–c) produced the following results:

(1) low-titre antibodies to peptides 526–549, 536–560, 546–570 and 562–585 (not shown) were found;
(2) the presence of antibodies to peptides 577–595 and 577–599 correlates ($P<0.01$) with the stage of disease, decreasing from 55% (11 out of 20) in CDC stage II to 7.7% (one out of 13) in CDC stage III and 20% (three out of 15) in CDC stage IV and from 70% (14 out of 20) in CDC stage II to 61.5% (eight out of 13) in CDC stage III and 20% (three out of 15) in CDC stage IV;
(3) peptide 589–618 was immunodominant in all groups of patients, in agreement with other reports [3,4,28,29];
(4) antibodies to the Cys-loop CSGKLIC correlate ($P<0.01$) with disease progression [(12 out of 20 (60%) in CDC stage II; six out of 13 (46%) in CDC stage III; five out of 15 (33%) in CDC stage IV)], as shown by the reactivity to peptide 602–625;
(5) The glycosylated region downstream of the Cys-loop is not strongly immunogenic in man: only low-titre antibodies to peptides 609–635 and 632–654 were found; however, significantly higher titres were found against peptide 621–645, suggesting that an epitope whose reactivity is dependent to some extent on peptide folding is present in this region;
(6) the presence of antibodies directed against residues 642–673 greatly decreases ($P<0.01$) with disease progression [nine out of 20 (45%) in CDC stage II, two out of 13 (15.4%) in CDC stage III and one out of 15 (6.7%) in CDC stage IV].

Observations on syncytia formation

The syncytia-inhibition assays demonstrated that: (1) the presence of fusion-inhibiting antibodies in sera did not correlate with stage of disease [five out of 20 (25%) syncytia-inhibiting sera in CDC stage II, four out of 13 (30.7%) in CDC stage III and three out of 15 (20%) in CDC stage IV (Table 1)]; (2) fusion-inhibiting activity in sera did not correlate with the

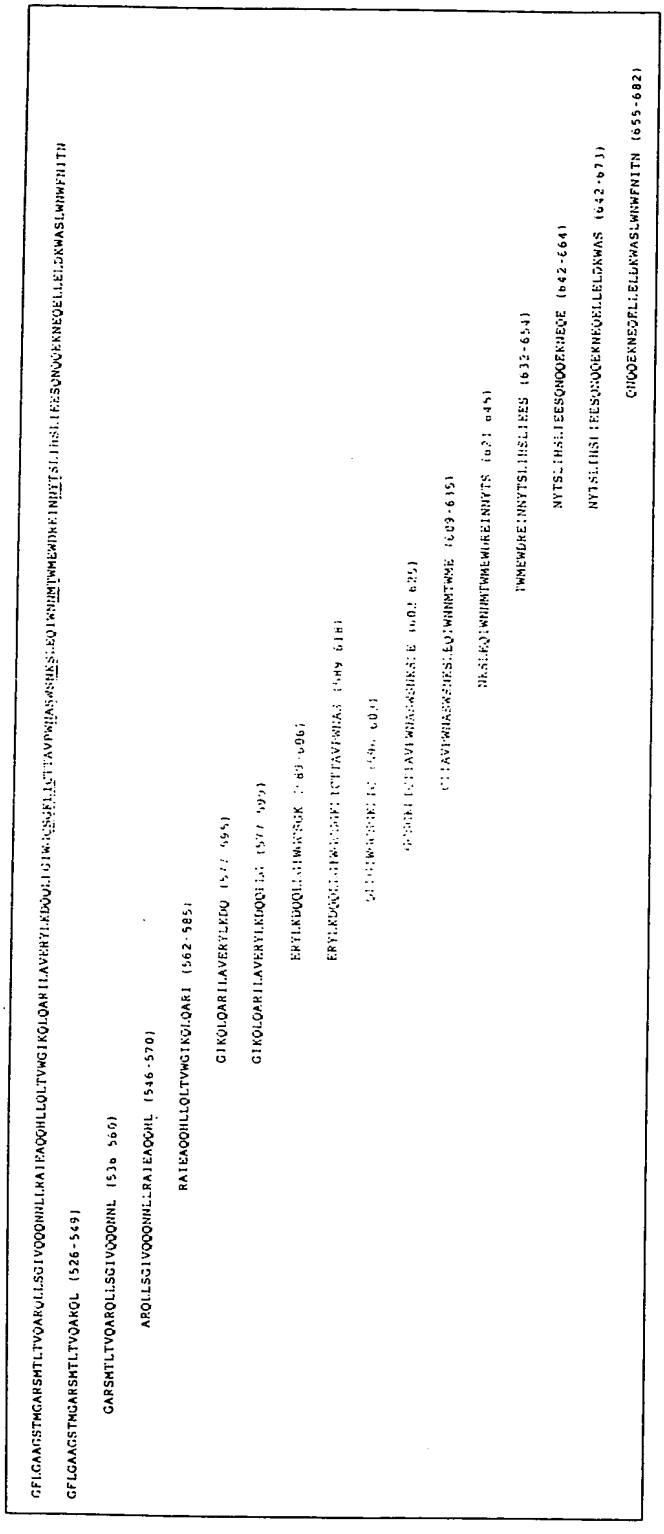

Fig. 1. Overlapping synthetic peptides spanning 526–682 residues of HIV-1 gp41 (*from the amino-acid sequence of the AIDS virus LAV reported by Wain-Hobson [24]). Potential glycosylation sites and the Cysteine loop between residues 603 and 609 are underlined.

Table 1. Syncytia inhibition by HIV-positive sera.

Serum	Dilutions		
	1:10	1:50	1:100
CDC stage II (20 sera)			
FAb	97*	94	90
GO	95	60	ND
LG	84	61	37
BM	100	53	40
PSb	100	94	90
15 sera	0	0	ND
CDC stage III (13 sera)			
VR	73	39	ND
RC	93	67	ND
PSc	100	39	ND
GGL	100	92	87
9 sera	0	0	ND
CDC stage IV (15 sera)			
FPA	77	40	ND
GG	82	39	ND
FAc	98	95	70
12 sera	0	0	ND

*Percentage inhibition of syncytia formation by sera at 1:10, 1:5 and 1:100 dilution. Percentage inhibition values at each dilution wer obtained as: 100−(number of syncytia in the presence of positiv serum/number of syncytia in the presence of a pool of 10 normal hu man sera at the same dilution) × 100. CDC, Centers for Disease Contro ND, not done.

presence of high-titre antibodies reacting with an of the peptides tested. This finding could imply tha most fusion-affecting antibodies are not directed to wards gp41.

Affinity purification of antibodies on Sepharose-linked peptides

Characterization and mapping of the eluted antibodies

To confirm that antibodies to defined sites of gp41 may be at least partially responsible for the fusion inhibiting activity exhibited by some sera, antibodies were affinity-purified on Sepharose-linked peptides and tested in the syncytia-inhibition assay. Peptides to be linked to cyanogen bromide-activated Sepharose were chosen on the basis of previous reports of the immunodominance [3,4,28,29] and/or the neutralizing capacity [1–7] of the corresponding antibodies.

Sera for affinity purification were selected on the basis of the titres exhibited in the inhibition of syncytia formation (Table 1).

The specificity of eluted antipeptide antibodies was tested in ELISA on both the relevant and control peptides and on gp120 recombinant protein. As shown in Table 2, purified antibodies reacted with the relevant gp41 peptide, but not with peptides of either the gp41 or gp120 envelope proteins, or with gp120 recombinant protein (strain IIIB). As expected, cross-reactivities were observed only for overlapping peptides.

Purified antibodies to the selected peptides were further assayed in Western blot on commercial HIV

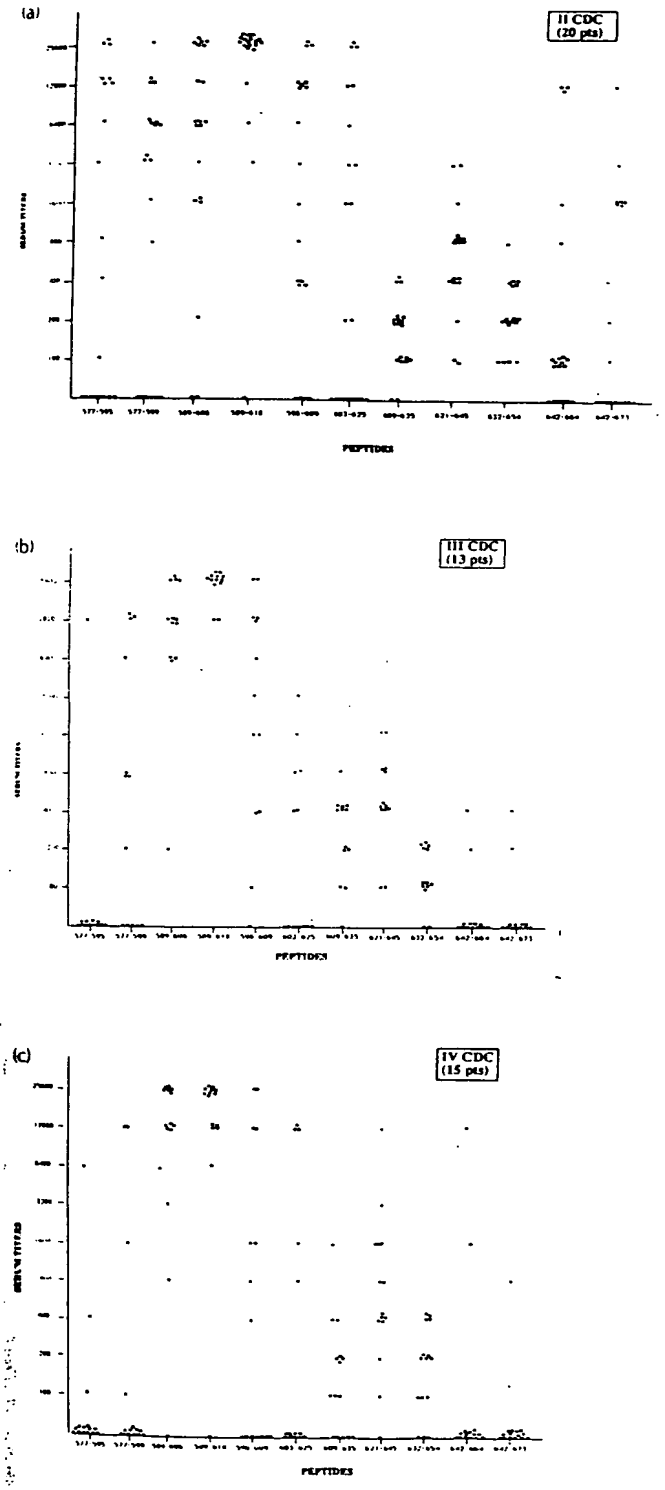

Fig. 2. Antibody titres against overlapping synthetic peptides covering the 577–682 region of gp41 transmembrane protein in HIV-positive sera from patients in Centers for Disease Control stages I (a), II (b) and III (c). Serum titres are calculated as the reciprocal of the highest dilution at which the ratio of absorbance corresponding to positive samples and absorbance corresponding to an equally diluted pool of 10 normal human sera was > 2.

strips (Sorin Biomedica). As shown in Fig. 3, purified antipeptide antibodies recognized the gp41 band, together with a 160 kD and a less intense 120 kD band, corresponding to the monomeric, tetrameric and trimeric forms of gp41, respectively [30,31]. Since the ELISA results on gp120 (shown in Table 2) were negative, the 120 kD band was interpreted as the trimeric form of gp41. The pattern of reactivity shown by affinity-purified human antibodies indicates that epitopes on gp41 multimers are more reactive, or simply more numerous, than epitopes on the monomeric form. Alternatively, the stronger staining at 120 and 160 kD could be explained by less monomer being transferred to Western blot strips than multimeric forms of gp41. Finally, we are unable to explain the significance of the additional unexpected bands observed with anti-589–618, 655–682 and 596–609.

Inhibition of syncytia formation by affinity-purified antibodies

As shown in Table 3, we purified syncytia-inhibiting antibodies on Sepharose-linked peptides of both the amino-terminal (577–618) and carboxy-terminal portions (655–682) of the gp41 region considered; in all cases, inhibition was shown to be dose-dependent. The data refer to representative single sera and each point represents the mean of two replicas. Antibodies immunopurified on the same peptide from different patients (irrespective of CDC stage) did not significantly differ in their syncytia-inhibition capacity.

Together with the epitope-mapping data shown in Table 2, these data strongly suggest the presence of fusion-relevant sites in both these regions.

As negative controls, the acid-eluted fraction of one normal human serum passed on Sepharose-linked peptides 589–618, the acid-eluted fraction of one positive serum passed on a Sepharose-linked unrelated peptide and normal human IgG purified on protein A Sepharose were tested in the syncytia-formation-inhibition assay. No syncytia-inhibiting activity could be demonstrated for antibodies purified on Sepharose-linked 546–570 and 642–664 peptides, although purifications were performed from selected sera exhibiting titres $\geq 1:3200$ of the corresponding antibodies.

Identification of discrete regions inside 25–30^{mer} synthetic peptides and tentative definition of their functional roles

Comparative analysis of the syncytia-inhibition capacity of purified antibodies (Table 3) and their fine reactivity in ELISA on the panel of synthetic peptides (Table 2) enabled identification of the amino-acidic residues defining sites critical for the fusion process.

Antibodies purified on both 577–595 and 577–599 peptides effectively inhibited syncytia formation, thus confirming involvement of the common 577–595 residues, comprising the already defined ARILAVERY neutralizing site [3], in the fusion process. Moreover,

Table 2. Mapping of defined epitopes within 25–30^{mer} synthetic peptides covering residues 577–682 of HIV-1 gp41 using affinity-purified antibodies.

Affinity-purified human antibody	ELISA reactivity on microtitre plates coated with peptide										
antipeptide	577–595	577–599	589–606	589–618	602–625	609–635	642–664	642–673	655–682	480–513*	gp120†
577–595	+	+	–	–	–	–	–	–	–	–	–
577–599	+	+ +	+	+	–	–	–	–	–	–	–
589–606	–	+	+ +	+ +	–	–	–	–	–	–	–
589–618	–	+	+ +	+ +	+ +	–	–	–	–	–	–
596–609	–	–	–	+	+	–	–	–	–	–	–
642–664	–	–	–	–	–	–	+ +	ND	–	–	–
642–673	–	–	–	–	–	–	–	+ +	+ +	–	–
655–682	–	–	–	–	–	–	–	+	+ +	–	–

Data were obtained in enzyme-linked immunosorbent assay (ELISA) on peptide coated (100 ng/well) microtitre plates. *Control peptide corresponding t residues 480–513 of gp120. †Recombinant protein from strain IIIB (ABT, Cambridge, Massachusetts, USA). +, > threefold over negative controls; + + > sixfold over negative controls. ND, not done.

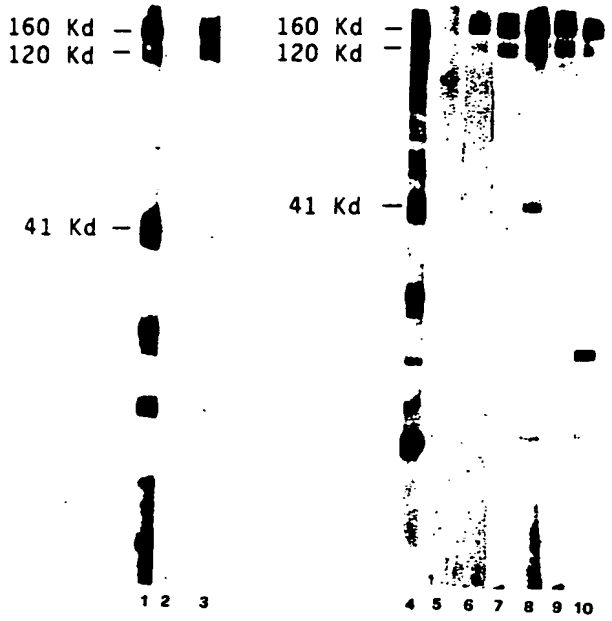

Fig. 3. Western blot reactivity of affinity-purified antibodies. The specificity of affinity-purified antibodies was analysed in Western blot on commercial HIV-1 test strips. Lane 1, human HIV-positive serum; lane 2, acid elution fraction from HIV-positive serum passed onto an unrelated control peptide Sepharose column; lane 3, affinity-purified antibodies antipeptide 577–599; lane 4, human HIV-positive serum; lane 5, acid elution fraction from HIV-positive serum passed onto an unrelated control peptide Sepharose column; lane 6, affinity-purified antibody antipeptide 577–595; lane 7, affinity-purified antibody antipeptide 589–606; lane 8, affinity-purified antibody antipeptide 589–618; lane 9, affinity-purified antibody antipeptide 655–682; lane 10, affinity-purified antibody antipeptide 596–609.

purified antibodies against 589–606 peptide (also effective in syncytia inhibition) were reactive in ELISA with peptides 577–599 and 589–618, but not with peptide 577–595, identifying the common 595–599 (QQLLG) residues as possibly involved in membrane fusion. Peptide 589–618 contains both the QQLLG site and the Cysteine loop (residues 603–609), already defined as an immunodominant, conformation-dependent site [4,8,9,10], but still not identified as neutralizable in man. As shown in Table 3, this peptide also contains fusion-relevant sites, as shown by the syncytium-inhibiting activity exhibited by the corresponding immunopurified antibodies. Moreover because of the fusion-inhibiting activity shown by antipeptide 596–609 antibodies, we can conclude that in addition to the QQLLG site, the Cys-loop also appears to be involved in membrane fusion.

Taken together, our data enable the identification of three distinct sites within region 577–609, defined by residues ARILAVERY, QQLLG and CSGKLIC, that are critical for the fusion process. The syncytia-inhibiting capacity of antipeptide antibodies is stronger when the antibodies are eluted from solid-phase peptides containing two of these sites. As shown in Table 3, 100% inhibition was achieved with 200 ng of antipeptide 577–599 and 589–618 antibodies (containing both ARILAVERY and QQLLG sites, or QQLLG and the Cys-loop, respectively), while twice this quantity (400 ng) was required to obtain the same effect with anti-577–595, 589–606 or 596–609 antibodies (containing ARILAVERY, QQLLG or Cys-loop sites, respectively).

Table 3. Inhibition of syncytia formation by human antibodies affinity purified from neutralizing sera.

Affinity-purified human antibody	Inhibition of syncytia formation in the presence of different amounts of purified antibodies (%)			
antipeptide	40 ng	100 ng	200 ng	400 ng
546–570	0	0*	0	0
577–595	0	34.7*	46.7	100
577–599	0	ND	100	100
589–606	0	15	46.6	100
589–618	0	10	100	100
596–609	0	22	60	100
642–664	0	0	0	0
655–682	0	0	100	100

*% inhibition values were obtained as: 100 − (number of syncytia in the presence of different amounts of purified antibodies/number of syncytia in controls) × 100. ND, not done.

As already observed, antibodies to ARILAVERY correlate strongly with disease progression, while antibodies to QQLLG (i.e., antibodies to residues 589–606, the only peptide containing QQLLG but neither ARILAVERY nor the Cys-loop) do not. Moreover, antibodies to CSGKLIC correlate with disease progression, as shown by reactivity to peptide 602–625. The lack of correlation demonstrated with peptides 596–609 and 589–618 (which also contain the Cys-loop) is probably due to their sharing the stage-independent immunodominant QQLLG residues.

As shown above (Figs 2a–c), the presence of antibodies directed against residues 642–673 considerably decreases with disease progression. The apparent beneficial role of such antibodies may be attributable to the effects of membrane fusion exhibited by anti-655–682 antibodies (Table 3). The fine mapping of this region using immunopurified antibodies (Table 2) identifies the critical site, corresponding to residues 664–673.

Discussion

Neutralizing antibodies have been demonstrated in infected individuals, although their significance in protecting against the pathogenic effects of the virus is not clearly understood. Moreover, the majority of the reported results on neutralizable sites have been produced in *in vitro* systems and most of the described neutralizing antibodies have been obtained in experimental animals.

Our aim was to define possible fusion-relevant sites of gp41 transmembrane protein identified by antibodies purified from sera of infected individuals on a panel of Sepharose-linked synthetic peptides and tested in a syncytia-inhibition assay.

Anti-gp14 antibodies with syncytium-blocking capacity were affinity-purified from all the fusion-inhibiting sera of our groups of patients. This result, together with the fine mapping of the fusion-relevant sites, demonstrates that both anti-gp120 antibodies and antibodies directed towards various sites of gp41 transmembrane protein may contribute to the syncytia-inhibition capacity exhibited by some positive sera.

Because of the conserved nature of region 577–599 [18–20] and the crucial structural role attributed to it [32], the identification of two fusion-critical sites corresponding to residues ARILAVERY and QQLLG gives further information about the secondary and tertiary structure of the *env*-encoded protein and should be taken into account in the design of a possible vaccine. It has been proposed that the Cysteine loop, together with residues upstream (and downstream) of amino-acid positions 603–609 may be involved in gp120–gp41 interactions [33]. Moreover, two anti-idiotypic monoclonal antibodies (M38 and 9G5A) that mimic two interacting sites of gp120 and gp41 have been described [26]. 9G5A is both syncytia-inhibiting and virus-neutralizing. Peptide mapping of both monoclonal antibodies has revealed that the QQLLG epitope recognized by 9G5A interacts with two homologous epitopes (KYK, corresponding to positions 490–492, and KAKRR, corresponding to positions 505–509 of gp120). The two gp120 epitopes are located immediately upstream and downstream of a large hydrophobic pocket containing six conserved residues shown to be crucial for the interaction and assembly of the gp120–gp41 glycoprotein complex [34]. Interestingly, the molecular model of Schulz *et al.* [33] proposes that the large hydrophobic pocket of gp120 is directly contacted by the gp41 Cys-loop.

Both interacting regions are highly conserved among retroviruses [35] and are highly immunogenic in man [36]. Moreover, deletion mutagenesis analysis has recently shown that residues upstream of the gp41 Cys-loop are involved in the interaction and assembly of surface and transmembrane *env*-encoded protein in Mason–Pfizer monkey virus [37]. Taken together, these observations suggest that the syncytia-inhibiting potential exhibited by purified human antibodies reactive with residues QQLLG should be ascribed to interference with the structural integrity of *env* transmembrane protein.

Although the spectrum of antibodies was distinct for each of the sera tested in ELISA on the panel of synthetic peptides, statistical analysis showed that antibodies to region 577–599 (peptides 577–595 and 577–599) and region 642–673 greatly decrease with disease progression. It has already been suggested that antibodies recognizing regions 583–599 and 658–682 may play a role in preventing progression to AIDS in HIV-1-infected individuals [5,28].

In conclusion, our approach has allowed the definition of discrete sites of gp41 that are critical for the fusion process and the purification of the corresponding antibodies from human positive sera. Definition of the *in vivo* functional relevance of the defined sites and of the protective role of the corresponding antibodies is required. This strategy may be relevant to the design of an effective *env* vaccine.

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